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Protective Role of Plastoquinone in the Early Stages of Second-Degree Thermal Skin Burn

Nadezhda I. Pashkevich[1], Ekaterina S. Pykhova[2], Alexander A. Ashikhmin[2], Daria V. Vetoshkina[2], Sergey S. Osochuk[1,a*], and Maria M. Borisova-Mubarakshina[2,b*]

[1]*Vitebsk State Order of Peoples' Friendship Medical University, 210009 Vitebsk, Republic of Belarus*

[2]*Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia*

[a]*e-mail: oss62@mail.ru* [b]*e-mail: mubarakshinamm@gmail.com*

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Abstract—Thermal burns of the skin are associated not only with local tissue alterations but also with the development of systemic disorders, which promote generalization of inflammatory processes. In particular, burn injury leads to an overproduction of reactive oxygen species, activation of free-radical oxidation, and lipid peroxidation. This study investigated the protective role of plastoquinone, a natural plant antioxidant, on the morphological condition of the skin and on the shape and aggregation of erythrocytes in rats with second-degree thermal burns. Thermal burn resulted in the decrease in epidermis thickness, increase in the number of hyperemic vessels, damaged hair follicles and sebaceous glands. Application of plastoquinone, incorporated into liposomes, onto the damaged skin had a protective effect on the skin structures; in the case of liposomes applied without plastoquinone, the protective effect was less pronounced. In addition, thermal burn altered the state of erythrocytes, leading to their deformation and aggregation. Plastoquinone in liposomes applied topically or administered intravenously showed a protective effect on erythrocytes comparable to that of ubiquinone, preventing the development of burn-induced erythrocyte shape alterations. However, only plastoquinone administered intravenously completely prevented erythrocyte aggregation, thus eliminating negative effects of the burn injury on the functional activity of erythrocytes, indicating the potential of plant-derived plastoquinone as an effective agent in burn injury management.

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Keywords: thermal burn, reactive oxygen species (ROS), liposomes, antioxidants, quinones, plastoquinone

INTRODUCTION

A thermal burn is an injury to the skin and underlying tissues, accompanied by complex pathophysiological changes including development of oxidative stress caused by enhanced generation of reactive oxygen species (ROS) and activation of inflammatory cascades [1]. ROS include superoxide anion radical ($O_2^{\bullet-}$) and its protonated form, hydroperoxyl radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), as well as hydroperoxides and radicals of organic molecules. All the cited ROS could be produced in animal cells, including $^1O_2$ in the reaction of spontaneous dismutation of $O_2^{\bullet-}$ [2]. Under conditions of burn trauma, the balance between the rate of ROS formation and the activity of antioxidant system in an organism is disrupted. Due to their high reactivity, ROS induce lipid peroxidation (LPO), causing damage to cell membranes and leading to cell dysfunction. ROS also cause oxidative modifications of amino acid residues in proteins and are capable of oxidizing carbohydrates and DNA [3]. In addition to local tissue damage, thermal burns negatively affect membranes of blood cells, erythrocytes membranes in particular. Structural changes have been reported in the erythrocytes passing through

* To whom correspondence should be addressed.

the inflammation region: deformability has been observed [4] as well as formation of cell aggregates shaped like a roll of coins (rouleaux) [5] due to modification of erythrocyte cytoskeleton protein, band 3, which could cause microvascular occlusion and hypoxia [6]. These changes occur mainly due to the effects of ROS produced by the damaged tissues, as well as by neutrophils (see below) [7]. Changes in the shape and aggregation of erythrocytes decrease their oxygen releasing capability, i.e. impair their oxygen transporting function [8].

Changed erythrocytes are mainly subjected to degradation through hemolysis. Hemoglobin released during erythrocyte degradation, i.e., extracellular "free" hemoglobin, is a contributing factor to the development of multiple organ dysfunction. Concentration of free hemoglobin in blood depends on the severity of skin burn injury [9]. In turn, free hemoglobin is a factor initiating production of ROS and proinflammatory cytokines [10] and, hence, as it circulates systemically in the bloodstream, contributes to secondary mitochondrial damage in the kidneys, heart, and muscle tissue [11], initiating the so-called secondary production of ROS or secondary oxidative stress [12].

Mitochondrial damage is accompanied by the release of mitochondrial DNA (mtDNA), which is most sensitive to the action of ROS due to the absence of histone proteins in its structure [13]. The presence of damaged mtDNA is closely associated with the induction of key proinflammatory cytokines, interleulin-6 (IL-6) in particular, as well as tumor necrosis factor-α (TNF-α) [14], which contribute to chronic inflammation and deterioration of tissue regeneration processes [15, 16]. Furthermore, it is known that mtDNA can enter the bloodstream, and an increased concentration of circulating mtDNA is associated with multiple organ dysfunction syndrome, which is one of the main causes of death of the patients with severe burn trauma [17, 18]. Thus, protection of erythrocytes against burn-induced deformation significantly reduces the risk of multiple organ dysfunction.

Considering destructive role of ROS in the development of multiple organ dysfunction in the case of burns described above, it seems relevant to investigate the use of antioxidant agents capable of minimizing the level of ROS and, thus, preventing primary and secondary tissue damage. Low molecular weight antioxidants are present in animal cells for protection against the endogenously formed ROS including glutathione, reduced pyridine nucleotides, bilirubin, uric acid, ubiquinone (UQ), as well as antioxidant enzymes: superoxide dismutases, catalase, peroxidases including peroxiredoxins. Some low molecular weight antioxidants such as ascorbic acid, tocopherols, flavonoids, and carotenoids are obtained through the diet. The majority of antioxidant systems are localized in the aqueous compartments of the cell, and not in the lipid hydrophobic membrane layers, where electron transport chains (in the inner mitochondrial membrane and in cytoplasmic membrane) are located, in which ROS could be generated with higher rates than in the aqueous phase. Many researchers emphasize the importance of protection of the cell membranes against the destructive actions of ROS for treating various diseases [19, 20]. In live cells utilization of ROS in the membranes is realized with participation of tocopherol and UQ [21, 22]. UQ in a reduced form, ubihydroquinone, neutralizes ROS responsible for LPO initiation, such as $O_2^{\bullet-}$ and $HO_2^{\bullet}$ [23]. Moreover, antioxidant function of UQ in LPO prevention is realized via reactions with LPO products as well – peroxide-, alkoxyl, and lipid radicals [24-26]. Protective effects of the exogenously administered UQ have been demonstrated in many diseases including burns [27-32]. The synthesized UQ has been actively investigated as a potential therapeutic agent in such pathologies as heart failure [33], neurodegenerative diseases [27, 28], as well as different types of cancer [30]. UQ investigations include both preclinical models and human clinical trials, including treatment of burns [31, 32]. UQ is also widely used in cosmetology as an ingredient with antioxidant properties.

At present, plastoquinone (PQ, component of the photosynthetic electron transport chain in chloroplasts) attracts more and more attention, together with other natural and synthetic analogues of PQ (see reviews [34-41]). This could be due to the fact that PQ activity in prevention of LPO was shown to be higher than activity of UQ and even tocopherol [42]. Taking into consideration high antioxidant activity of PQ, a number of synthetic derivatives of PQ have been developed and their effectiveness was tested both *in vitro* and *in vivo*. The most effective ones were compounds known as Skulachev ions that are composed of a synthetic analogue of PQ, decylplastoquinone, linked to triphenylphosphonium for mitochondria targeting. Effectiveness of Skulachev ions was observed at very low concentrations due to their high partition coefficient between the aqueous and hydrophobic phases, as well as ability of decylplastoquinone to be reduced to decylplastohydroquinone in the complexes I and II of the mitochondrial respiratory chain [43]. The developed synthetic derivatives of PQ continue to be investigated [44]. In addition, information on other synthetic variants of PQ is available in the literature, including various derivatives of 1,4-benzoquinone, which lack or have a modified isoprenoid fragment. Such halogenated and non-halogenated compounds demonstrate pronounced cytotoxic properties with regard to cancer cells [45, 46] and also exhibit antimicrobial [47, 48] and antifungal effects [49].

At the same time, use of these synthetic derivatives of PQ in treating burns has not been investigated in detail.

Neutrophils represent an important source of free radicals in the early stages of thermal burn damage; they initiate the so-called respiratory burst due to enhanced production of $O_2^{\bullet-}$ by NADPH oxidases of the plasma membrane (see review [1]). In this regard, use of synthetic derivatives of PQ targeting mitochondria could be not very reasonable. Considering all the above, it seems more logical to use PQ without mitochondria targeting in this case.

However, there is a considerable lack of data in the literature on protective effects of isolated and purified plant PQ. In the course of our previous studies antioxidant properties of PQ in plant leaves have been investigated (see review [50]). Antioxidant function of PQ, similar to UQ, is realized by PQ in the fully reduced form (plastohydroquinone (PQH_2)); PQH_2 effectively neutralizes $O_2^{\bullet-}$ and $HO_2^{\bullet}$, as well as $^1O_2$ (see review [50]), that protects thylakoid membrane against LPO and prevents pigment bleaching [51]. Neutralization of $O_2^{\bullet-}$ by the PQH_2 molecules is assumed to be one of the main reactions demonstrating its antioxidant activity in a photosynthesizing cell [52]; rate constant of this second order reaction is estimated to be ~10^7 M^{-1} s^{-1} [53]. Rate constant of the reaction with $^1O_2$ is even higher – ~10^8 M^{-1} s^{-1} [54], however, under stress conditions $O_2^{\bullet-}$ is generated in leaves with higher rates than $^1O_2$, hence, the probability of PQH_2 reaction with $O_2^{\bullet-}$ is significantly higher; significant formation of $^1O_2$ is typical only for more severe conditions, which are not so common in nature (see review [35, 55]). The possibility of PQH_2 reaction with H_2O_2 has been also demonstrated [56, 57]. However, as emphasized by the authors [56], reaction rate constant in this case is rather low ~10^4 M^{-1} s^{-1}. Despite the low-rate constant, H_2O_2 effectively oxidizes the PQ pool in thylakoids in the dark after illumination [58].

Hence, PQ is a promising natural antioxidant compound with high capability for neutralization of ROS. Moreover, PQ, similar to UQ, exhibits highly pronounced membranotropic properties inducing ordering of membrane structure [59]. Owing to these properties, PQ appears to be a promising candidate protecting cellular structures in burn trauma. This study aims to evaluate protective properties of PQ isolated and purified from higher plants under conditions of thermal skin burn damage in laboratory animals. Possible mechanisms of cytoprotective effects of PQ in burns are considered in the present study including its effect on inflammatory response and oxidative stress.

Traditionally liposomes are employed for delivery of lipophilic compounds. It is known that benzoquinones with a long side isoprenoid chain, such as PQ and UQ, are effectively incorporated into liposomes and preserve their antioxidant activity [60-63]. Compositions of membrane lipids and of fatty acids in cells change in burns, that includes decrease of the content of cholesterol esters, phospholipids, amount of essential (ω-3 and ω-6) polyunsaturated fatty acids (PUFA), as well as oxidation of PUFA [64, 65]. Considering this fact, use of liposomes due to their composition and size could facilitate replenishment of the content of essential ω-3 and ω-6 PUFA and cholesterol. In our previous study on skin thermal burns in rats, liposomes with varying content of phosphatidylcholine were used; it was found out that liposomes, prepared from lecithin with 90% of phosphatidylcholine, display more pronounced protective activity in comparison with the liposomes, prepared from lecithin with 26% of phosphatidylcholine [66]. Therefore, goal of this study was evaluation of protective properties of PQ incorporated into liposomes prepared from cholesterol and lecithin, containing 90% of phosphatidylcholine.

MATERIALS AND METHODS

Enzyme-linked immunosorbent assay of proinflammatory cytokines IL-6 and TNF-α content in human blood. Study was conducted using blood cells of volunteer donors (males and females) aged 28-38 years. To determine cytokine levels, freshly drawn venous blood stabilized with heparin was used. For the experiments, 100 μl of blood was diluted 10-fold in RPMI-1640 medium (PanEco, Russia) and incubated in 12-well plates (Nunc, Denmark) at 37°C in a 5% CO_2 atmosphere for 6 h. Total volume of incubation mixture was 1 ml. Hexa-acetylated lipopolysaccharide (LPS) from Gram-negative bacterium *Escherichia coli* (*E. coli*) O55:B5 was used as an inducer of TNF-α and IL-6 [67]. Samples were incubated in the presence of: 100 ng/ml of LPS, or 1 μM of PQ, or 10 μM PQ, or 1% ethanol, or without any added compounds (control group). After incubation plates were centrifuged at 1500*g* for 15 min using a microplate rotor centrifuge centrifuge (BioSan, Latvia). The obtained blood plasma was diluted with a RPMI-1640 medium: for TNF-α assay – 5-fold, for IL-6 – 35-fold. Cytokine production was evaluated with an enzyme-linked immunosorbent assay using reagent kits from Vektor-Best (Russia). Optical density was recorded with a STAT FAX 3200 microplate reader (Awareness Technology Inc., USA) at 450 nm. Cytokine concentration was calculated based on calibration curve constructed with standard samples provided by the manufacturer.

Laboratory animals. Experimental studies with laboratory animals were conducted based on collaboration agreement between the Vitebsk State Order

of Peoples' Friendship Medical University (VSMU) and Pushchino Scientific Center for Biological Research, Russian Academy of Sciences, in the research laboratory of VSMU.

Seven-month-old white male outbred rats of the *Rattus Muridae* line (n = 74 for 3-h experiments, and n = 78 for 24-h experiments) with mass 190-364 g were used in the study. Animals were kept under standard conditions in a vivarium (temperature 22 ± 2°C and relative humidity 50-65%, 12-hour light cycle, free access to feed and water). Animals were not fed during the night before experiment.

Isolation and purification of PQ from higher plants. PQ was isolated according to the method described previously [68-71] with some modifications to obtain PQ in amount sufficient for conducting experiments. In particular, the step of preliminary chlorophyll removal from the extract using aluminum oxide was modified. Freshly-cut leaves of amaranth/spinach/pea with removed stems were homogenized in a Braun 4184 blender (Braun, Czech Republic) in a buffer (50 mM Hepes-KOH (pH 7.6), 20 mM NaCl, 5 mM $MgCl_2$). Homogenate was filtered through two layers of nylon fabric and extracted with acetone at a ratio of 1 : 2 (1 part of filtrate (100-200 ml) and 2 parts of acetone) and 30 ml of hexane followed by 5-min incubation for layer separation. Next upper hexane phase was collected and 30 ml of hexane were added followed by extraction and sample collection. Volume of the hexane mixture was reduced to 1 ml in a rotary evaporator. The concentrate was applied onto an aluminum oxide chromatography column pre-equilibrated with hexane. Elution was carried out first with hexane : acetone 95 : 5, and the PQ-containing fraction was collected until the appearance of an orange carotenoid band. Then, a second eluent (hexane : acetone, 90 : 10) was applied, and the PQ-containing fraction was further collected. The pooled fraction was concentrated in a rotary evaporator and analyzed at 40°C using Shimadzu HPLC system (Shimadzu, Japan) with a reversed phase C18 column (Waters Spherisorb 5 μm ODS2, size 4.6×250 mm, Waters, USA). Acetonitrile: ethanol mixture 3 : 1 (V/V) was used as a mobile phase, flow rate was 1,5 ml/min. Retention time for PQ was 22.4 min. PQ concentration was evaluated spectrophotometrically based on absorption at 255 nm. Extinction coefficient was 18 $mM^{-1}\cdot cm^{-1}$ [59]. To confirm the compound structure a high-resolution mass spectrometer Orbitrap Elite ETD (Thermo Scientific, Germany) was used. The obtained spectrum was compared with theoretical molecular weight of PQ and literature data confirming identification of the isolated compound as PQ.

Preparation of liposomes and incorporation of PQ or UQ into liposomes. Liposomes were prepared based on lecithin containing 90% of phosphatidylcholine (PanReac AppliChem, Spain, Germany) and cholesterol (Sigma, USA) at a ratio 5 : 1 according to the method described by Khoshneviszadeh et al. [72]. Lecithin (7.78%) and cholesterol (1.5%) were dissolvedin chloroform and methanol at a ratio 3 : 1. Ethanol solution of PQ or UQ (Sigma, USA) to achieve final concentrations 5 or 50 μM was added to this mixture; in the case of preparation of empty liposomes same volume of ethanol was added to liposomes, as in the case of PQ or ubiquinone solutions. Thin lipid film was formed using vacuum evaporation in a rotary evaporator at 45°C. The formed film was slowly re-suspended in a 0.1 M potassium-phosphate buffer with pH 8.0 for topical application, or in isotonic 0,9% NaCl solution for intravenous administration. Obtained liposome solutions were extruded through an Avanty 400-nm laboratory mini-extruder (Avanti Polar Lipids, USA). Liposome size was measured by dynamic light scattering using with a Zetasizer Nano (Malvern Instruments, UK). The mean size of liposomes without PQ was 580.23 ± 137.72 nm, and with PQ – 695.24 ± 167.93 nm (Fig. 1). There were no statistically significant differences between the sizes of obtained liposomes (p-value 0.1564 (t)).

Modeling of thermal burn. Animals were separated into 8 groups, which were examined 3 and 24 h after inducing burn:

1. Control – intact animals, not subjected to any exposures (for 3-h experiments n = 12, for 24-h experiments n = 12);

2. Sham control – animals subjected to manipulations similar to those in experimental procedures, but without inducing thermal burn (for 3-h experiments n = 10, for 24-h experiments n = 10);

3. Thermal burn without treatment — animals with induced second-degree thermal burns without following treatment (for 3-h experiments n = 10, for 24-h experiments n = 10);

4. Thermal burn and treatment with empty liposomes – animals with thermal burn, which were treated with liposomes without active agent topical application (for 3-h experiments n = 8, for 24-h experiments n = 9);

5. Thermal burn and 50 μM PQ, topical application – animals with thermal burn treated with topical application of liposomes with 50 μM PQ (for 3-h experiments n = 8, for 24-h experiments n = 9);

6. Thermal burn and 5 μM PQ, intravenous administration – animals with thermal burn treated with intravenous administration of liposomes with 5 μM PQ (for 3-h experiments n = 10, for 24-h experiments n = 10);

7. Thermal burn and 50 μM UQ, topical application – animals with thermal burn treated with topical application of liposomes with 50 μM UQ (for 3-h experiments n = 8, for 24-h experiments n = 9);

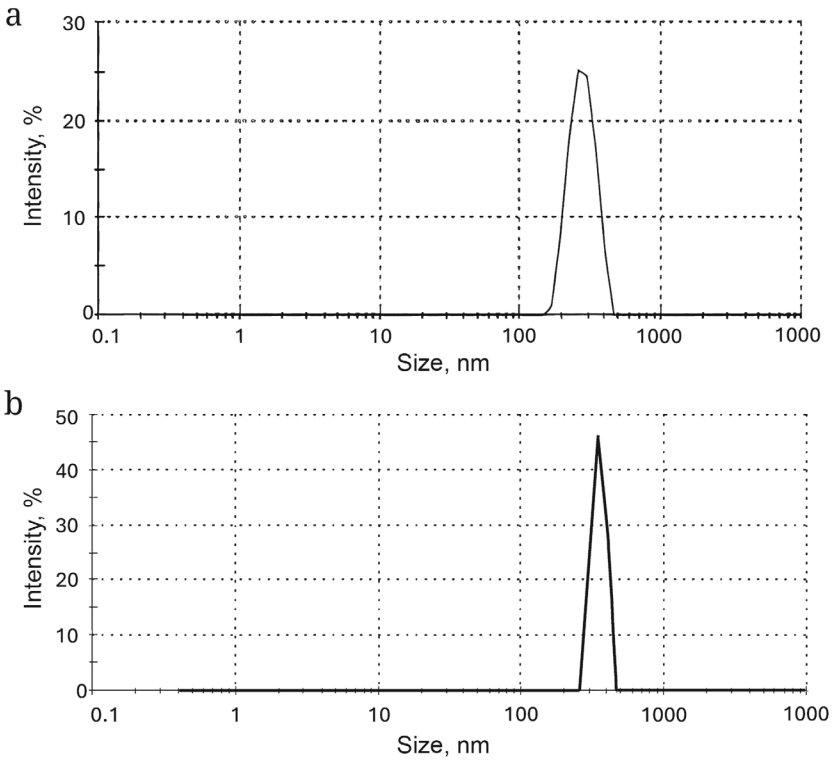

Fig. 1. Size distribution of the obtained liposomes. a) Empty liposomes; b) liposomes with incorporated PQ.

8. Thermal burn and 5 μM UQ, intravenous administration – animals with thermal burn treated with intravenous administration of liposomes with 5 μM ubiquinone (for 3-h experiments n = 8, for 24-h experiments n = 9).

To model burn, animals were anesthetized by ketamine according to approved recommendations [73-75]. Thermal injury was introduced by applying a metal strip heated to 150°C to the shaved dorsal skin for 4 min, which morphologically corresponded to second-degree burn according to The International Classification of Diseases (ICD), 10th revision (burns of degree IIIA according the previously used classification) [76]. An instrument for burn modeling was constructed in OAO KB Display (Vitebsk, Republic of Belarus) in the framework of collaboration agreement with the Vitebsk State Order of Peoples' Friendship Medical University. Burn was introduced on a back of an animal in the preselected region. Burn surface area was 30% of the total body surface area (TBSA). To calculate TBSA, the Meeh formula (1) was used:

$$S = k \times W^{2/3}, \quad (1)$$

where S – total body surface area, cm^2; k – Meeh constant equaling to 9.46 [77]; W – body mass of an animal, kg.

Immediately after thermal injury, the test compounds were administered either topically or intravenously. Animals in group 4 were treated with 0.45 ml of suspension of empty liposomes per burn area. Animals in groups 5 and 7 were treated with 0.45 ml of suspension of liposomes with incorporated 50 μM PQ or UQ, respectively. After application, the liposomal suspension was polymerized in air, forming a dense dry film. To prevent interactions and traumatization of burn surface animals were housed individually in separate cages. Animals in groups 6 and 8 were immediately after burn initiation treated with lateral tail vein injection (0.45 ml) of liposomal forms of PQ or UQ at concentration 5 μM, administered into the lateral tail vein, using isotonic 0.9% NaCl as a diluent.

Animals were euthanized by decapitation using a laboratory guillotine following induction of ether anesthesia. Euthanasia was carried out 3 or 24 h after modeling thermal burn. Blood was collected into heparinized tubes for further analysis during time period of preservation of spinal automatism. Biopsy samples from the burn injury area were obtained using a sharp instrument and a technique that minimized tissue deformation, for further histological examination.

Assessment of erythrocyte count and morphology. The number and morphological alterations of erythrocytes were evaluated using in a Goryaev counting chamber under a Leica DM 2000 microscope equipped with an 8× objective and a 10× eyepiece (Leica Microsystems, Germany) [78]. To examine number and shape of erythrocytes whole blood was used,

which was diluted 200-fold in 0.9% NaCl (to prevent hemolysis in the tube).

Assessment of erythrocyte aggregation. The number of aggregated erythrocytes was determined according to established methods using a Goryaev counting chamber and a Leica DM 2000 microscope equipped with a 20× objective (Leica Microsystems, Germany) [79]. Whole blood was used diluted 200-fold with 0.9% NaCl (to prevent hemolysis in the tube).

Determination of haptoglobin concentration. Determination of haptoglobin concentration in blood plasma was carried out using commercial ELISA kit (cat. no. E-EL-R0473, Elabscience, China) according to the manufacturer's instructions. The analysis was performed using theVityaz F300TP immunoassay analyzer (Republic of Belarus).

Preparation of skin biopsies and morphological analysis. Skin samples were fixed in 10% neutral buffered formalin at room temperature prior to histological processing. After standard dehydration in a graded series of ethanol solutions with increasing concentration and paraffin embedding, samples were washed with running water for several hours, next, sections with 3-4 μm thickness were prepared from block samples with a Leica RM 125 rotary microtome. Histological samples were stained with hematoxylin and eosin according to the standard technique and examined in a Leica DM 2500 microscope, 10× eyepiece, 20× and 40× objectives (Leica Microsystems, Germany), equipped with a Leica DFC 320 digital camera. The number of damaged elements was counted in 10 fields of view, from which average numbers of skin elements were calculated.

Measurement of spontaneous chemiluminescence. Whole blood samples collected after decapitation with heparin added as an anticoagulant were examined. An aliquot (0.1 ml) of a heparinized blood was placed into a cuvette. Spontaneous chemiluminescence was recorded over a period of 2 min using a Lum-100 device (Disoft, Russia). The area under the chemiluminescence curve was used as the analytical parameter.

Statistical data processing. Statistical analysis of the data was carried out with the help of R version 4.0.5 (2021-03-31), and Origin 2021 software packages. The distribution of the studied variables was assessed using the Shapiro–Wilk test; in the case of Gaussian distribution parametric statistical methods were used for comparison, otherwise non-parametric methods were used. Pairwise comparisons were carried out based on the Student's and Mann–Whitney–Wilcoxon tests. Multiple comparisons were carried out with the help of ANOVA (in the case of heterogeneity of dispersions of the investigated parameters Welch correction was used) or Kruskal–Wallis H test. *Post-hoc* analysis was carried out using Tukey's test or Kruskal–Wallis H test in Dunn modification with correction for multiple comparisons according to the Benjamini–Yekutieli or Holm–Bonferroni methods. Differences were considered statistically significant at $p < 0.05$. When presenting data as boxplots: boxes (rectangles) represent the interquartile range (lower border corresponds to 25th percentile and the upper one – 75th percentile), horizontal line is a median, square – arithmetic mean, whiskers – minimum and maximum values not considering outliers, diamond-shaped symbols – outliers.

RESULTS

Investigation of the effects of PQ addition on production of proinflammatory cytokines IL-6 and TNF-α in human blood. It is known that appearance in blood of even small concentrations of LPS, which possesses very high proinflammatory activity, could result in the development of endotoxin shock increasing production of early proinflammatory cytokines such as TNF-α and IL-6 [67]. Therefore, the level of TNF-α and IL-6 production serves as a marker of proinflammatory activity.

To evaluate content of TNF-α and IL-6 blood plasma obtained from whole human blood was used. As can be seen in Fig. 2, the addition of LPS from *E. coli* (100 ng/ml) to whole blood resulted in significant, more than 100-fold, increase of production of TNF-α. To establish possible proinflammatory effect of PQ, blood was first incubated with PQ at final concentration 1 or 10 μM. Considering that PQ was added in ethanol solution (resulting in a final ethanol concentration of 1%), in the first step effect of ethanol addition on production of TNF-α was evaluated; it was revealed that ethanol in the used concentration did not exhibit proinflammatory effect. It was established in the following experiments that PQ in the investigated concentrations also did not induce production of TNF-α by the cells in whole blood (Fig. 2), which indicated absence of manifestations of proinflammatory activity of the plant-derived PQ.

In addition, it was shown in our study that addition of LPS to whole human blood significantly activated IL-6 synthesis (Fig. 3). The obtained data confirm the fact that the used LPS indeed has clearly pronounced proinflammatory activity. Similar to the case of TNF-α measurements, addition of PQ at both concentrations did not cause enhanced production of IL-6; the level of IL-6 production was similar to the one measured in the control samples and in the samples with ethanol.

Thus, the results obtained in investigation of the level of TNF-α and IL-6 in the samples of whole human blood provide reliable evidence that PQ does not exhibit proinflammatory activity and may be further

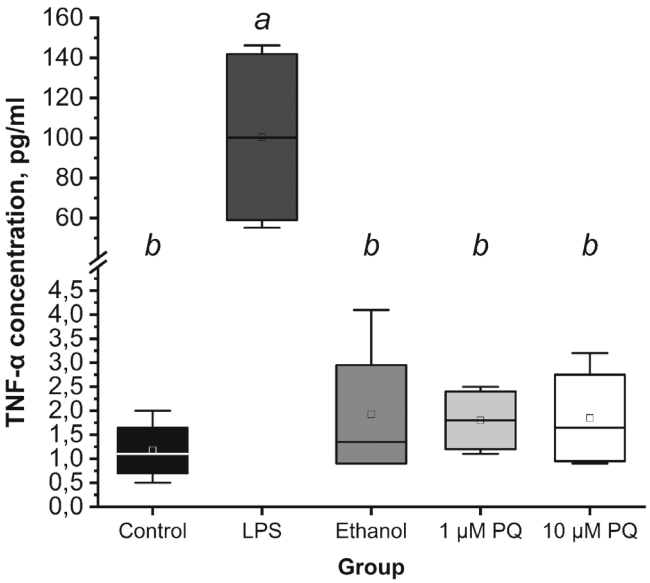

Fig. 2. Effect of PQ on production of TNF-α in human blood plasma. LPS – hexa-acylated lipopolysaccharide of Gram-negative bacterium *E. coli* O55:B5. LPS concentration in incubation medium was 100 ng/ml (see "Materials and Methods" section). Mean values from two independent experiments are presented, for each experiment blood was obtained from two donors. Statistically significant differences between the groups were evaluated with one-way ANOVA followed by *post-hoc* comparison according to Holm–Bonferroni method. Columns designated by different letters (*a*, *b*), differ significantly between each other with $p < 0.05$.

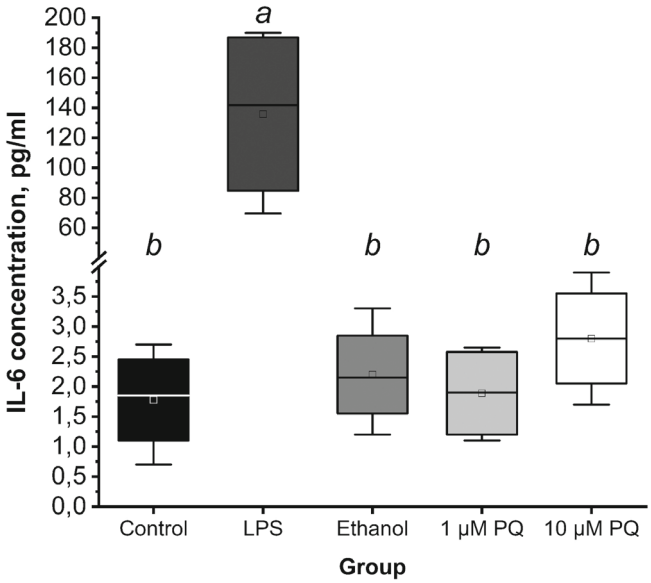

Fig. 3. Effect of PQ on production of IL-6 in human blood plasma. LPS – hexa-acylated lipopolysaccharide of Gram-negative bacterium *E. coli* O55:B5. LPS concentration in incubation medium 100 ng/ml (see "Materials and Methods" section). Mean values from two independent experiments are presented, for each experiment blood was obtained from two donors. Statistically significant differences between the groups were evaluated with one-way ANOVA followed by *post-hoc* comparison according to Holm–Bonferroni method. Columns designated by different letters (*a*, *b*), differ significantly between each other with $p < 0.05$.

investigated as a protective agent in thermal burns induced in laboratory animals.

Effect of PQ on morphological parameters of skin in second-degree thermal burns. In the next step of our study modeling of thermal burns affecting 30% of total body surface was carried out using laboratory animals (rats); and potential protective effect of PQ on the state of skin was evaluated. Outbred white rats were used in experiments. For burn modeling the shaved skin of animals was exposed to heat (4 min, 150°C) using a special device; the resulting injuries morphologically corresponded to the second-degree burns (see Materials and Methods section). Immediately after burn injury the affected skin region was treated with either empty liposomes or liposomes with incorporated PQ. One day (24 h) after the burn injury, morphology of skin biopsies from experimental animals were examined.

Thermal burn of animals (group 3) resulted in the statistically significant decrease, almost 2-fold, of the epidermis thickness, stratum corneum thickness, and epidermis thickness excluding the stratum corneum, compared both to the control animals (group 1) and in comparison with the animals subjected to sham control (group 2), i.e. animals subjected to all same manipulations, but without exposure to heat (Table 1). The revealed changes are associated with capacity of burns to cause necrosis of epidermis and denaturation of proteins in epidermal layer [80]. The animals of group 3 have increased number of hyperemic vessels, damaged hair follicles, and sebaceous glands in comparison with the animals of groups 1 and 2 (Fig. 4, Table 1).

Liposomes without PQ (group 4) did not display any protective effect on epidermis thickness, thickness of the stratum corneum, and thickness of the epidermis without stratum corneum (Table 1). However, numbers of hyperemic vessels, as well as number of damaged hair follicles were lower after application of empty liposomes on the skin of animals in comparison with the animals of group 3. No damaged sebaceous glands were observed in animals of group 4. The presented data are in agreement with the data reported in our previous study obtained during comparison of the effects of liposomes with different content of phosphatidylcholine in treatment of burn injuries, where similar regularities were revealed for the liposomes produced based on lecithin containing 90% phosphatidylcholine [66].

To investigate effects of PQ on the state of skin and its associated elements such as hair follicles and sebaceous glands, as well as on the vessels in dermis

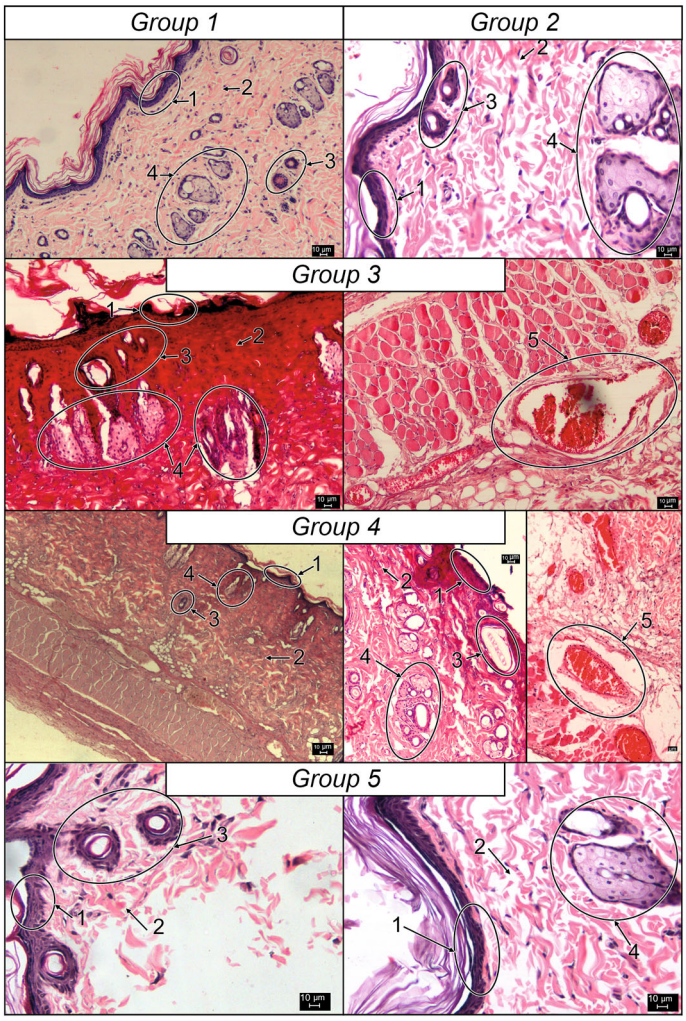

Fig. 4. Typical skin micro-preparations of the studied animal groups (stained with hematoxylin and eosin; objectives – 20× and 40×). *Group 1* – control (n = 12); *Group 2* – sham control (n = 10); *Group 3* – thermal burn without treatment (n = 10); *Group 4* – thermal burn and liposomes without active ingredient (n = 9); *Group 5* – thermal burn and PQ 50 μM, topical application (n = 9). One typical micro-preparation each is presented for *Group 1* and *Group 2*. Labels: 1 – epidermis, 2 – dermis, 3 – hair follicles, 4 – sebaceous glands, 5 – hyperemic blood vessel.

during thermal burn, PQ purified from plant leaves biomass was incorporated into the lipid phase of liposomes as described in the Materials and Methods section. As can be seen from the results presented in Table 1, topical application of liposomes with 50 μM PQ (group 5) decreased the degree of damage of skin layers after thermal burn: decrease of the total thickness of epidermis was less pronounced than in groups 3 and 4. The thickness of epidermis without the stratum corneum in the group 5 was the same as in groups 1 and 2. A positive, but less pronounced effect was observed for the stratum corneum. Moreover, use of liposomes with incorporated PQ protected hair follicles from damage: no damaged hair follicles were observed in animals of group 5 as in the cases of groups 1 and 2 (Table 1).

Hence, thermal burn of skin layers caused statistically significant changes in all investigated parameters. Liposomes without PQ, by replenishing the PUFA deficit, decreased the number of hyperemic vessels and prevented damage to the sebaceous glands. The use of liposomes with incorporated PQ facilitated preservation of dermis structure, and completely prevented damage to hair follicles.

Effect of PQ on the state of erythrocytes and oxidative destruction in the blood of experimental animals with second-degree thermal burn. In the next stage of the study the effect of skin burn on the shape and aggregation of erythrocytes was investigated (Fig. 5), as well as the level of spontaneous chemiluminescence in the blood of experimental animals.

Skin thermal burn significantly increased number of erythrocytes with morphological alterations (group 3) both in comparison with the group 1 and the group 2 (Fig. 6, a, b); at the same time, no changes

Table 1. Effect of the second-degree thermal burn on morphological parameters of the skin of animals 24 h after thermal burn injury, and evaluation of protective effect of empty liposomes or liposomes with PQ on the investigated parameters (details on group composition are presented in "Materials and Methods section")

Group 1	*Group 2*	*Group 3*	*Group 4*	*Group 5*
Epidermis thickness, μm				
29.42 ± 2.75 *a*	29.83 ± 3.33 *a*	12.33 ± 4.83 *b*	12.25 ± 4.03 *b*	23.08 ± 4,10 *c*
Thickness of the stratum corneum of the epidermis, μm				
14.00 ± 1.65 *a*	15.92 ± 2.47 *a*	5.92 ± 2.84 *b*	5.00 ± 2.95 *b*	10.50 ± 2.65 *ab*
Thickness of the epidermis (excluding stratum corneum), μm				
15.42 ± 2.07 *a*	13.92 ± 1.68 *a*	6.42 ± 2.27 *b*	7.25 ± 1.71 *b*	12.58 ± 1.78 *a*
Number of hyperemic vessels				
0.00 ± 0.00 *a*	0.00 ± 0.00 *a*	14.50 ± 4.72 *b*	7.75 ± 1.22 *c*	6.42 ± 2.78 *c*
Number of damaged hair follicles				
0.00 ± 0.00 *a*	0.00 ± 0.00 *a*	6.58 ± 1.51 *b*	3.42 ± 0.90 *c*	0.00 ± 0.00 *a*
Number of damaged sebaceous glands				
0.00 ± 0.00 *a*	0.00 ± 0.00 *a*	6.42 ± 1.00 *b*	0.00 ± 0.00 *a*	0.00 ± 0.00 *a*

Note. *Group 1* – control (n = 12); *Group 2* – sham control (n = 10); *Group 3* – thermal burn without treatment (n = 10); *Group 4* – thermal burn and liposomes without active ingredient (n = 9); *Group 5* – thermal burn and PQ 50 μM, topical application (n = 9). Values are presented as an arithmetic mean ± standard deviation of the mean (M ± SD). Statistical processing was performed using non-parametric method (Kruskal–Wallis H test in Dunn modification with correction for multiple comparisons according Benjamini–Yekutieli). Values denoted with different letters (*a*, *b*, *c*) are statistically significantly different with $p < 0.001$. Designation of *ab* type indicate a group, which does not differ significantly either from *a*, or from *b* at $p = 0.001$.

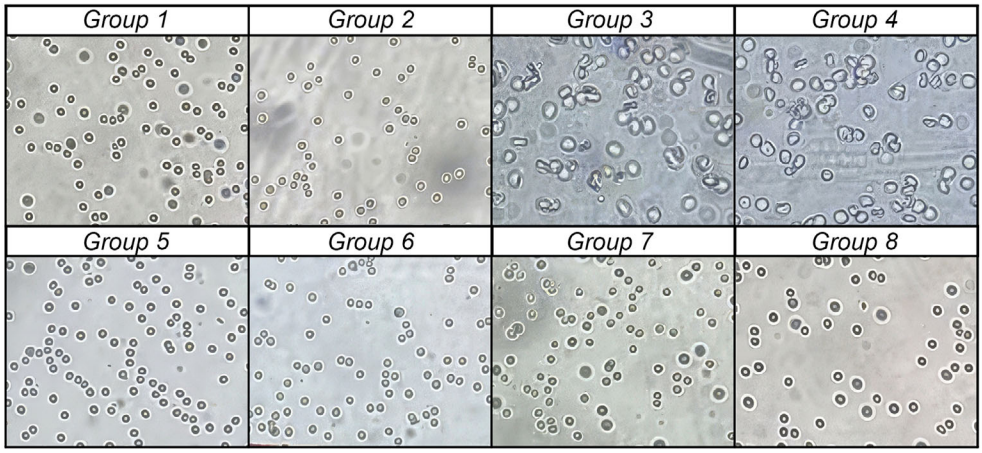

Fig. 5. Typical images demonstrating the condition of erythrocytes in experimental animals 24 h after burn injury, and evaluation of the protective effect of empty liposomes, liposomes with PQ or UQ on the studied parameters. Images were obtained using a Leica DM 2000 microscope. *Group 1* – control (n = 12); *Group 2* – sham control (n = 10); *Group 3* – thermal burn without treatment (n = 10); *Group 4* – thermal burn and liposomes without active ingredient (n = 9); *Group 5* – thermal burn and 50 μM PQ, topical application (n = 9); *Group 6* – thermal burn and 5 μM PQ, intravenous (n = 10); *Group 7* – thermal burn and 50 μM UQ, topical application (n = 9); *Group 8* – thermal burn and 5 μM UQ, intravenous (n = 9).

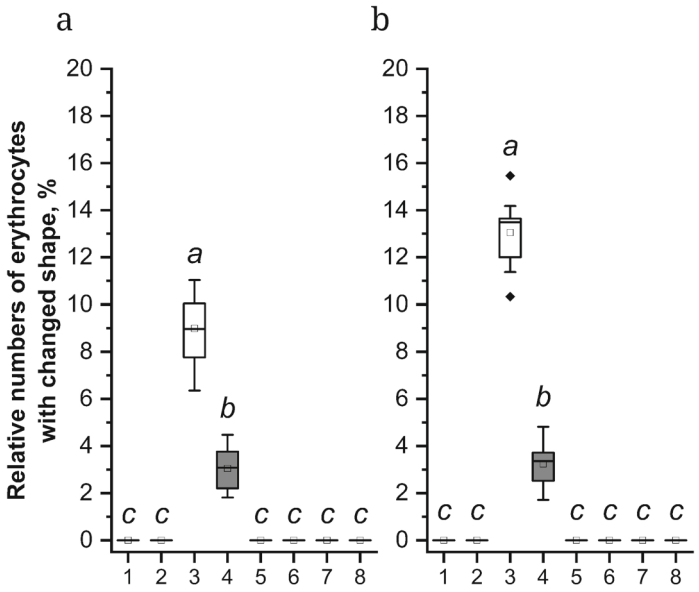

Fig. 6. Relative numbers of erythrocytes with morphological alterations in the rat blood 3 h (a) and 24 h (b) after thermal burn injury. Statistical significance of the differences between the groups were evaluated by one-way ANOVA with *post-hoc* comparison using Holm–Bonferroni method. *Group 1* – control (for 3-h n = 12, for 24-h n = 12); *Group 2* – sham control (for 3-h n = 10, for 24-h n = 10); *Group 3* – thermal burn without treatment (for 3-h n = 10, for 24-h n = 10); *Group 4* – thermal burn and liposomes without active ingredient (for 3-h n = 8, for 24-h n = 9); *Group 5* – thermal burn and 50 μM PQ, topical application (for 3-h n = 8, for 24-h n = 9); *Group 6* – thermal burn and 5 μM PQ, intravenous (for 3-h n = 10, for 24-h n = 10); *Group 7* – thermal burn and 50 μM UQ, topical application (for 3-h n = 8, for 24-h n = 9); *Group 8* – thermal burn and 5 μM UQ, intravenous (for 3-h n = 8, for 24-h n = 9). Columns denoted with different letters (*a*, *b*, *c*) differ significantly with $p < 0.05$.

in the total number of erythrocytes were observed (data not shown). The deformed erythrocytes were observed both 3 and 24 h after the burn injury (Fig. 6, a, b); furthermore, their amounts were comparable. Application of empty liposomes (group 4) reduced the number of erythrocytes with morphological alterations. In the case of the group 5, this parameter practically was the same as in groups 1 and 2, i.e. no any erythrocytes with morphological alterations were detected. In the case of intravenous administration of physiological solution containing liposomes with 5 μM PQ, erythrocytes with morphological alterations were also not observed. The obtained results indicate the protective effect of PQ on erythrocytes in thermal burns.

Similar data were obtained in our previous study modeling ultraviolet-induced skin burn in the animals with burn area of 30% of the total body surface. Application immediately after burn of liposomes with incorporated PQ (10 and 100 μM, topical application) or intravenous administration of liposomes with 5 μM PQ prevented development of morphological changes in the erythrocytes observed 24 h after ultraviolet burn injury (Patent of the RF (11) 2 819 761) [81].

Moreover, in our experiments we compared the effects of PQ with the effects of UQ which is a native component of animal cells, and is widely used at present as an antioxidant agent. It was revealed that topical application of UQ in liposomes (group 7) or its intravenous administration (group 8) at the same acting concentrations as in the case of PQ also completely prevented erythrocyte deformation already 3 h after thermal burn (Fig. 6a).

The obtained data indicate that thermal burn facilitates emergence of erythrocytes with morphological alterations. Empty liposomes are capable of reducing the number of deformed erythrocytes, and use of liposomes with antioxidants can completely prevent erythrocyte deformation in the second-degree thermal burn.

As noted in the Introduction, changes in erythrocyte morphology occur due to enhanced formation of ROS in the inflammation area and initiation of oxidative destruction, including activation of LPO. Mechanisms of these processes involve free-radical oxidation and are accompanied by an increase in spontaneous chemiluminescence of blood [82]. Therefore, intensity of spontaneous chemiluminescence in the blood of experimental animals was assessed in all investigated groups. Three hours after burn injury, i.e., under conditions, when a large number of deformed erythrocytes was observed (see above), increase of spontaneous chemiluminescence of the blood in animals was detected (Fig. 7), which is in agreement with the literature data [82].

Topical application of liposomes without antioxidants (group 4) decreased the level of spontaneous

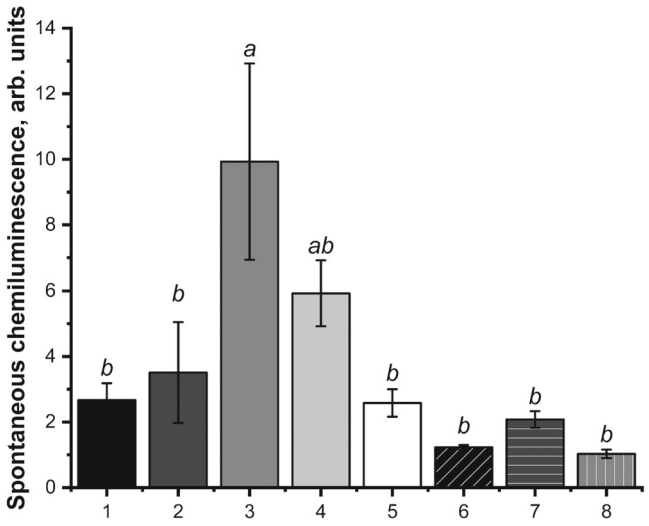

Fig. 7. The level of spontaneous chemiluminescence in the blood of animals 3 h after burn injury. Data are presented as the arithmetic mean (denoted by bars with standard error). Statistically significant differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Holm–Bonferroni *post-hoc* comparison. *Group 1* – control (n = 12); *Group 2* – sham control (n = 10); *Group 3* – thermal burn without treatment (n = 10); *Group 4* – thermal burn and liposomes without active ingredient (n = 8); *Group 5* – thermal burn and 50 μM PQ, topical application (n = 8); *Group 6* – thermal burn and 5 μM PQ, intravenous (n = 10); *Group 7* – thermal burn and 50 μM PQ, topical application (n = 8); *Group 8* – thermal burn and 5 μM UQ, intravenous (n = 8). Bars denoted by different letters (*a*, *b*) are significantly different from each other at $p < 0.05$. The designation *ab* indicates an intermediate group that is not significantly different from either group *a* or *b* ($p > 0.05$).

chemiluminescence in blood (Fig. 7). Topical application and intravenous administration of liposomes with PQ (groups 5 and 6) or UQ (groups 7 and 8) provided the level of spontaneous chemiluminescence already 3 h after burn injury, which was comparable with the level observed for the groups 1 and 2. The obtained data indicate that both PQ and UQ suppress oxidative destruction caused by the second-degree burn.

In addition to affecting shape of erythrocytes, burns also initiate erythrocyte aggregation [83]. Experimental facts have been presented in the literature indicating that the ability of erythrocytes for aggregation is determined by their ζ-potential, which quantitatively characterizes the surface negative charge of erythrocytes determining their resistance to aggregation [84]. Decrease of the ζ-potential level facilitates erythrocyte aggregation and their assembly into rouleaux, which interferes with oxygen release from erythrocytes causing tissue hypoxia [8]. Analysis of the level of erythrocyte aggregates demonstrated (Fig. 8), that after 3 h number of aggregates increased in comparison with the control animals and animals from the group subjected to sham control and remained at the increased level even 24 h after inducing thermal burn, that indirectly implies decrease of their ζ-potential and worsening of oxygen transport function for at least one day after burn.

Topical application of empty liposomes without antioxidants decreased significantly 3 and 24 h after burn the number of erythrocyte aggregates. Topical application of PQ (group 5) and UQ (group 7) at concentration 50 μM resulted in the significant decrease of the number of aggregated erythrocytes, however, this level did not differ statistically significantly from the level observed in the group 4 and remained higher than in groups 1 and 2. In the case of intravenous administration of 5 μM UQ (group 8) aggregation of erythrocytes decreased significantly only after 24 h in comparison with the group 4, but aggregation did not cease completely. Only intravenous administration of PQ (5 μM) (group 6) completely prevented erythrocyte aggregation: aggregated erythrocytes were observed neither after 3 h nor after 24 h.

The obtained results allow confirming capability of PQ and UQ to reduce erythrocyte aggregation likely due to preservation of their ζ-potential. Data presented in Fig. 8 demonstrate that intravenously administered PQ exhibits the highest efficacy in preventing erythrocyte aggregation, which suggests potential for the use of PQ in the complex therapy of burns.

Considering that the changes in the shape and aggregation of erythrocytes are accompanied with the increase of hemolysis and release of free hemoglobin as a factor of generalization of burn injury and the role of PQ in preventing the pathogenetic events, haptoglobin concentration in plasma was additionally measured by ELISA in the animals with burns treated with PQ (groups 5 and 6). It is known that haptoglobin binds free hemoglobin [85], hence, decrease of its concentration indicates presence of hemolysis. Amount of haptoglobin was significantly higher in animals of the group 5, and was insignificantly higher in the case of group 6 in comparison with the animals after burn (group 3): in the group of animals with burns this parameter was 71.3 ± 12.5 g/L (n = 10, p = 0.0260), and in the animals treated with liposomal PQ by topical application – 104.6 ± 27.4 g/L (n = 12, p = 0.0304), and in the animals with intravenous administration – 88.2 ± 25.7 g/L (n = 15, p = 0.3508). The obtained data demonstrate less intensive hemolysis in the animals treated with liposomal PQ administered on skin. These results were obtained with the blood samples collected 24 h after burn and do not reflect dynamics of the changes of haptoglobin/hemoglobin concentration in the blood of animals, which requires further investigation.

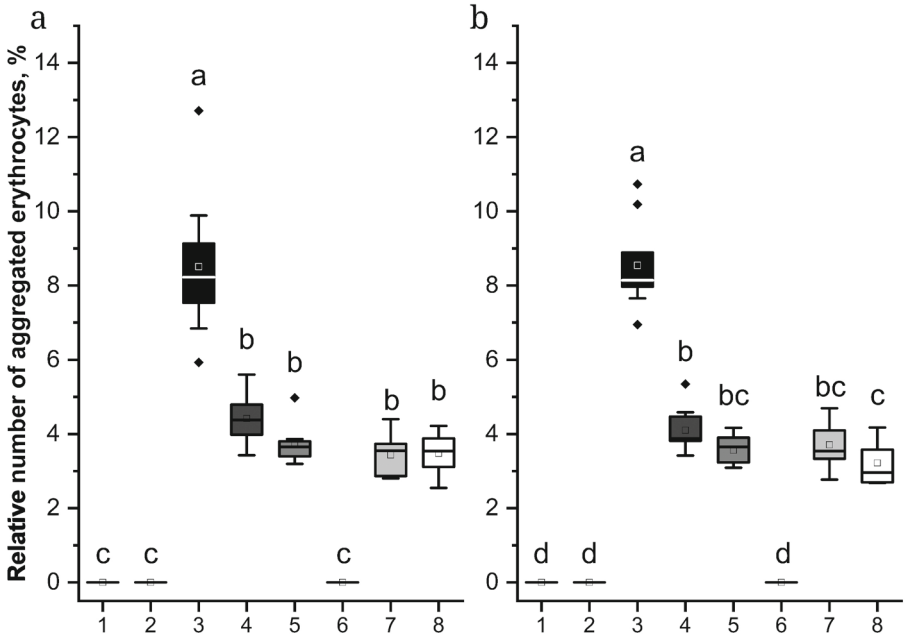

Fig. 8. Changes in the number of aggregated erythrocytes in the blood of animals 3 h (a) and 24 h (b) after thermal burn injury. Statistically significant differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Holm–Bonferroni *post-hoc* comparison (for details on group composition, see the legend to Fig. 6). Columns denoted with different letters (*a*, *b*, *c*, *d*) differ significantly with $p < 0.05$. The designation *bc* indicates an intermediate group that is not significantly different from either group *b* or *c* ($p > 0.05$).

DISCUSSION

Protective effects of PQ isolated from plant cells in treatment of burns in animals was demonstrated for the first time in this work. For this purpose, the method of isolation and purification of PQ from leaves of higher plants was optimized to obtain quantities sufficient for conducting tests (see "Materials and Methods" section). Thermal burn causes changes in morphological structure of skin layers: thickness of epidermis is decreased including thickness of basal layer and epidermis thickness without basal layer, as well as hair follicles and sebaceous glands are damaged (Table 1). Empty liposomes without PQ facilitate preservation of hair follicle structure, which mainly is, most likely, due to phospholipid composition of the liposomal particles [86], in particular due to the high content of fatty acids in phosphatidylcholine including lineic, palmitic, and oleic acids. Lecithin containing 90% of phosphatidylcholine used for preparation of liposomes in our study contained fatty acids (% of total content of fatty acids): C16:0 – 13.63; C18:0 – 3.68; C18:1n9c – 11.36; C18:2n6c – 62.88; C18:3n3 – 6.12 [66]. Data are available in the literature that linoleic and oleic acids are capable of decreasing intensity of acute inflammation due to inhibition of synthesis and activity of proinflammatory cytokines [87, 88], and palmitic acid displays high stability and resistance against oxidation, and also could be used as an alternative source of energy under conditions of energy deficit [89].

The noticed in our study decrease of the number of hyperemic vessels after the use of empty liposomes could be also associated with replenishing of the PUFA deficit. Hence, liposomes prepared from lecithin, containing 90% phosphatidylcholine, decrease the manifestation of the inflammatory reaction in the zone of burn probably due to stabilization of cellular membranes, which is in agreement with the data reported in our previous study [66].

Use of PQ incorporated into lipid phase of the liposomes results in much less pronounced damages of skin layers after thermal burns: epidermis in the group of animals threated with these liposomes was less damaged in comparison with the epidermis of animals subjected to burns and animals with burns treated with empty liposomes, numbers of damaged hair follicles decreased (Table 1). It is possible that the antioxidant and membrane-stabilizing properties of PQ (see "Introduction" section) underlie the decrease of damages of skin layers and skin-associated elements, such as hair follicles.

Considering that free-radical oxidation also involves PUFA, it is possible that PQ is capable of potentiating activity of liposomes associated with compensation of PUFA deficit thus protecting the newly replenished PUFA against further oxidation decreasing activity of production of proinflammatory lipoxins from PUFA of ω-9 series [90]. In the process PQ itself does not exhibit anti-inflammatory effects, because its use in the whole blood samples is not

accompanied by the increase of production of TNF-α and IL-6 (Figs. 2 and 3), that indicates lack of activation of classic proinflammatory signaling pathways when this antioxidant is used.

PQ affected positively not only skin layers during burns, but also state of blood cells, shape of erythrocytes, in particular. Despite its plant origin, PQ demonstrated protective effect comparable with the effect of UQ, preventing development of burn-induced changes of erythrocyte shape (Fig. 6). It must be mentioned that liposomes without PQ decreased the number of deformed erythrocytes, however, the liposomes with PQ and UQ maintained this number at the level typical for the control animals. Deformation of erythrocytes is also associated with activation of free-radical oxidation and accompanying reactions [65, 91, 92]. The obtained result could be due to the combined action of the used liposomes and antioxidants allowing not only to replenish PUFA and cholesterol deficit in the damaged tissues, but also to effectively neutralize ROS, which is in agreement with the results obtained during investigation of spontaneous chemiluminescence (Fig. 7). Moreover, the revealed increase of haptoglobin content after topical application of PQ indicates decrease of degree of hemolysis [92], which allows suggesting decrease of the rate of generalization of the disorder caused by burns. Lack of the effect on the content of haptoglobin in the case of intravenous administration of PQ likely indicates that the effective prevention of hemolysis in burn injury could be achieved only in the case of existence of protective effect in the skin layers.

Therefore, it was proved in our study that the use of liposomes with antioxidants, PQ or UQ, in particular, administered either by topical application or intravenously, could completely prevent changes in erythrocyte shape after second-degree burns (Fig. 6) and prevent disruption of their oxygen transport function decreasing the degree of hemolysis and release of hemoglobin as a factor facilitating generalization of inflammatory process and multiorgan failure. It could be suggested that prevention of hemolysis, as one of the early and critical stages in burn pathogenesis, could significantly reduce negative effects of burn injury, however, in order to prove that prevention of hemolysis is a vital step in the interruption of the chain of pathogenetic events in skin burns, further investigations are required.

Another manifestation of negative influence of thermal burn on blood parameters is erythrocyte aggregation, which, similar to the change of their shape also negatively affects their oxygen transport function [8]. The results of our investigation demonstrate that the plant-derived PQ has clearly pronounced protective properties exceeding protective effects of ubiquinone; intravenous administration of PQ incorporated into liposomes completely protects erythrocytes against aggregation in burn injury (Fig. 8), which, likely, allows to maintain erythrocyte functions at the level typical for healthy animals. Based on the information presented above, it could be suggested that for maximum protection in treating burns combination of intravenous administration and topical application of preparations could be recommended.

Due to its lipophilic nature, PQ is capable of integration into the membrane lipid bilayer of the damaged cells. Furthermore, incorporation of PQ into composition of liposomes enriched with PUFA facilitates more efficient delivery of antioxidant to the damaged skin areas. Through integration into the membrane redox system, PQ, likely, could be reduced to PQH_2 effectively neutralizing such ROS as $O_2^{\bullet-}$, $HO^{\bullet}$, $^1O_2$, and others (see “Introduction” section), thus preventing development of LPO and attenuating consequences of the second-degree thermal burn. Despite the absence of direct data on the involvement of PQ in mitochondrial chain in animals, such effects seem plausible based on the known properties of quinones [93, 94]. The level of reduced UQ in plasma membrane could be maintained by a number of enzymes: DT-diaphorase [95], cytochrome b5 reductase [96], NADPH:quinone reductase [63], glutathione reductase [97], lipoamide dehydrogenase [98], and thioredoxin reductase [99]. Majority of these enzymes are NADH- and NADPH-dependent, and it was shown that incubation of the isolated liver cell membrane with NADH increased the fraction of reduced UQ in them [100]. Hence, exogenous UQ and PQ are capable of being reduced by the native membrane components effectively neutralizing ROS in plasma membrane. The obtained results help to define direction of further research in the use of PQ in composition of liposomes as an agent for early intervention in treatment of thermal burns preventing hemolysis and release of free hemoglobin, which mediates generalization of inflammation process. The possibility of using PQ at the later stages of burn injury requires further investigation.

Synthetic PQ analogues, as is well-known, are used as therapeutic agents exhibiting anti-inflammatory, antibacterial, anticancer, and other properties; however, as a rule, synthetic PQ analogues have shorter side chain, which limits their solubility in membranes and manifestation of antioxidant properties in prevention of LPO in membranes [35]. Considering lack of proinflammatory effect of PQ added to whole blood observed in our study (Figs. 2 and 3), as well as its clear protective effect under condition of oxidative stress, PQ could be used in treatment of pathological states associated with the increased concentrations of ROS.

CONCLUSION

Use of liposomes in composition of burn-treatment mediations results in reduction of negative consequences of thermal skin injury, and use of the plant-derived PQ incorporated into liposomes almost completely eliminates consequences of the second-degree burns. PQ does not induce a proinflammatory response *in vitro* and effectively prevents morphological changes in the skin layers, as well as change of the shape and aggregation of erythrocytes in the second-degree burn injury. Therefore, application of PQ incorporated into liposomes could be considered as a method for protection of erythrocytes against deformation and hemolysis. In addition, it ensures the preservation of the thickness of the epidermis and prevents damage to hair follicles and sebaceous glands.

Abbreviations. IL-6, interleukin 6; LPO, lipid peroxidation; LPS, lipopolysaccharide; mtDNA, mitochondrial DNA; PQ, plastoquinone; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α; UQ, ubiquinone.

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Contributions. M. M. Borisova-Mubarakshina, S. S. Osochuk – setting goals and objectives of the experimental study; N. I. Pashkevich, E. S. Pykhova, A. A. Ashikhmin, D. V. Vetoshkina – conducting experiments; N. I. Pashkevich, E. S. Pykhova, D. V. Vetoshkina, S. S. Osochuk, M. M. Borisova-Mubarakshina – discussion of the obtained results; N. I. Pashkevich, E. S. Pykhova – writing draft of the paper; N. I. Pashkevich, E. S. Pykhova, D. V. Vetoshkina, M. M. Borisova-Mubarakshina – editing text of the paper.

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Ethics approval and consent to participate. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. For the enzyme-linked immunoassay of IL-6 and TNF-α in human blood, the blood of voluntary donors was used.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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